Small-Molecule Multidrug Resistance–Associated Protein 1 Inhibitor Reversan Increases the Therapeutic Index of Chemotherapy in Mouse Models of Neuroblastoma

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Abstract

The multidrug resistance–associated protein 1 (MRP1) has been closely linked to poor treatment response in several cancers, most notably neuroblastoma. Homozygous deletion of the *MRP1* gene in primary murine neuroblastoma tumors resulted in increased sensitivity to MRP1 substrate drugs (vincristine, etoposide, and doxorubicin) compared with tumors containing both copies of wild-type *MRP1*, indicating that MRP1 plays a significant role in the drug resistance in this tumor type and defining this multidrug transporter as a target for pharmacologic suppression. A cell-based readout system was created to functionally determine intracellular accumulation of MRP1 substrates using a p53-responsive reporter as an indicator of drug-induced DNA damage. Screening of small-molecule libraries in this readout system revealed pyrazolopyrimidines as a prominent structural class of potent MRP1 inhibitors. Reversan, the lead compound of this class, increased the efficacy of both vincristine and etoposide in murine models of neuroblastoma (syngeneic and human xenografts). As opposed to the majority of inhibitors of multidrug transporters, Reversan was not toxic by itself nor did it increase the toxicity of chemotherapeutic drug exposure in mice. Therefore, Reversan represents a new class of nontoxic MRP1 inhibitor, which may be clinically useful for the treatment of neuroblastoma and other MRP1-overexpressing drug-refractory tumors by increasing their sensitivity to conventional chemotherapy. [Cancer Res 2009;69(16):6573–80]

Introduction

Intrinsic or acquired multidrug resistance is one of the major causes of treatment failure in human malignancy. Among genes that mediate multidrug resistance, the clinical relevance of multidrug resistance–associated protein (MRP1) has been best established in the aggressive childhood malignancy, neuroblastoma. MRP1 acts as an ATP-dependent efflux pump for the transport of organic anions, glutathione-, glucuronate-, or sulfate-conjugated drugs, or unconjugated drugs in concert with free glutathione (1, 2), including the chemotherapeutic agents vincristine, doxorubicin, and etoposide. The down-regulation of MRP1 activity in neuroblastoma cells by *MRP1* antisense mRNA (3) or by treatment with MRP1 reversal agents (4) results in increased sensitivity to cytotoxic drugs. More importantly, high *MRP1* expression in primary neuroblastoma at diagnosis is strongly associated with poor patient outcome (5, 6). Thus, identification of inhibitors of this multidrug transporter is of clinical importance. Although, in recent years, several MRP1 inhibitors have been identified, the number of compounds close to or entered into clinical trials is limited (e.g., sulindac; ref. 7).

In general, multidrug resistance mediated by multidrug transporters, such as P-glycoprotein (Pgp) and MRP1, results in resistance to a broad spectrum of structurally unrelated drugs, and classic inhibitors are substrates of these transporters, reversing resistance by competitive inhibition. Indeed, many of the first- and second-generation Pgp inhibitors were such classic inhibitors and it is believed that this mechanism of action contributed to their failures (8). During the course of our efforts to identify inhibitors of p53 (9), we identified a new class of Pgp modulator that did not globally reverse multidrug resistance but rather altered the substrate specificity of the transporter (10). This study raised the possibility of rational control over cell sensitivity to drugs using these types of modulators that went beyond the classic competitive inhibition of transporter function. Thus, a small-molecule library selected around these new Pgp modulators represented a potential source of novel modulators or inhibitors of other multidrug transporters, such as MRP1, with unique mechanisms of action.

Through our screening efforts, we have identified six structural scaffolds that can effectively inhibit MRP1 function with the most active compounds clustered within the pyrazolopyrimidine scaffold, the focus of the studies presented here. Reversan, one of the most potent pyrazolopyrimidines identified to date, when used in combination with either vincristine or etoposide to treat neuroblastoma *in vivo*, increased tumor sensitivity to these conventional drugs with no increased toxicity. Therefore, we have identified a safe small-molecule inhibitor of MRP1 that may have clinical potential in the treatment of neuroblastoma and other cancers that overexpress MRP1.

Materials and Methods

Chemicals

2-Nitrophenyl-β-D-galactopyranoside, DMSO, methylene blue, daunorubicin, doxorubicin, vincristine, etoposide, cisplatin, paclitaxel, probenecid,

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

C.A. Burkhart and F. Watt contributed equally to this work. A.V. Gudkov and M. Haber are co-corresponding authors on this work.

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and verapamil were purchased from Sigma. Cyclosporin A was purchased from Fluka. MK571 was obtained from Calbiochem. Cyclophosphamide was purchased from Baxter Health Care. Difloxacin was obtained from Abbott Laboratories. PAK104P was a kind gift from Prof. Shin-Ichi Akiyama (Kagoshima University). Matrigel basement membrane matrix was obtained from BD Sciences. Isoflurane was purchased from Abbott Australasia.

Cell Lines

MCF7 and its derivatives [MCF7/VP (11) and MCF7/VP-LacZ], HCT116 and BE(2)-C and its Pgp-overexpressing derivative BE(2)-C/CHCb(1) (kindly provided by Dr. Barbara Spengler, Fordham University), were maintained in DMEM supplemented with 10% fetal bovine serum. SK-RC45 was maintained in RPMI supplemented with 10% fetal bovine serum and 25 mmol/L HEPES. All media were supplemented with glutamine and antibiotics. MDCKII, MDCK/MRP1, MDCK/MRP2, and MDCK/MRP3 cells and the human embryo kidney HEK293, HEK/MRP4, and HEK/MRP5 cells (kindly provided by Dr. Piet Borst, Netherlands Cancer Institute) were maintained as described previously (12–14).

Screening of Chemical Libraries

Screening A. A library of 2,300 structural analogues (ChemBridge) assembled around Pgp modulators identified previously (10) was screened for MRP1 modulators using a cell-based readout system based on previously described readout systems that used a p53-responsive LacZ reporter to detect the presence of DNA damage mediated by doxorubicin (Supplementary Fig. S1), a substrate for multidrug transporters (9, 10). In the current study, the human breast cancer cell line MCF7/VP, which has amplified *MRP1* and negligible Pgp, were stably transduced with a p53-responsive LacZ reporter to create readout cell line, MCF7/VP-p53-LacZ. For screening, MCF7/VP-p53-LacZ cells (2×10^4 per well) were seeded into 96-well plates. The next day, cells were treated with 0.9 μmol/L doxorubicin in the presence of library compounds (10 μmol/L). DMSO, doxorubicin alone (0.23-1.8 μmol/L), and 0.9 μmol/L doxorubicin in the presence of verapamil (2.5-20 μmol/L) served as controls. The following day, reporter activity was measured as described previously (9). "Hits" were defined as any compounds that increased reporter activity to a level equal to or greater than the level achieved with 20 μmol/L verapamil in the absence of direct induction of the p53-responsive reporter by the compounds themselves (i.e., in the absence of doxorubicin).

Screening B. A library of 299 compounds (ChemBridge) with ≥90% structure similarity to active compounds representing six prominent scaffolds (Supplementary Fig. S2) identified in screening A was screened as described above.

Drug Accumulation Assay

Select hit compounds were tested for their ability to modulate cellular accumulation of daunorubicin, a fluorescent MRP1 substrate. MCF7 and MCF7/VP cells were pretreated with each of the hit compounds for 10 min before the addition of daunorubicin (0-1.8 μmol/L) for 100 min. Verapamil and MK571 served as positive controls. Following washing with PBS, intracellular daunorubicin was eluted with 70% ethanol and the fluorescence measured using 485/535 nm filters (Wallac, Perkin-Elmer).

Cytotoxicity Assays

To determine the effect of the hits on drug sensitivity, MCF7/VP cells were treated for 18 h with a concentration range of MRP1 substrate drugs (doxorubicin, vincristine, and etoposide) or nonsubstrate drugs (cisplatin and paclitaxel) in the presence or absence of hit compounds. Following the incubation, medium was replaced and cells were allowed to grow for an additional 48 h. Cells were then stained with 0.5% methylene blue. Eluted dye was measured at 600 nm. Fold sensitization was defined as the ratio between the IC_{50} of drug alone and the IC_{50} of drug plus compound. The same methodology was used to evaluate the effect of the hits on the drug sensitivity of human tumor cell lines [BE(2)-C, HCT116, and SK-RC45] and for the evaluation of the effect of modulators on other drug transporters.

Protein Isolation and Western Analysis

Total cell lysates from BE(2)-C, HCT116, SK-RC45, and MCF7/VP were prepared using radioimmunoprecipitation assay buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Sigma). Proteins were separated on 4% to 15% Tris-HCl gels (Bio-Rad) and transferred to nitrocellulose. Blots were incubated overnight with a MRP1-specific antibody (MRPr1 1:5,000; Alexis) in TBS containing 0.5% (w/v) skim milk and 2 h with a goat anti-rat secondary antibody (1:10,000; Amersham Biosciences). To control for gel loading, blots were reprobed for 2 h with an α-tubulin-specific antibody (1:2,000, clone DM1A; Sapphire Biosciences) in TBS+0.05% Tween 20 and 1 h with a sheep anti-mouse secondary antibody (1:10,000; Amersham Biosciences). Proteins were visualized using SuperSignal reagent (Progen Biosciences).

Care and Maintenance of Mice

The generation and maintenance of the human *MYCN* (*hMYCN*) transgenic mouse model of neuroblastoma have been described previously (15, 16). BALB/c mice were obtained from Taconic Farms. Nude mice (*nu/nu*) of BALB/c background were obtained from the Biological Resource Facility of the University of New South Wales. All experimental procedures involving mice were approved by the University of New South Wales Animal Care and Ethics Committee according to the Animal Research Act (1985) and the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (1997) or the Institutional Animal Care and Use Committee of the Cleveland Clinic Foundation according to the USPHS Policy on Humane Care and Use of Laboratory Animals.

Establishment of Neuroblastoma Allografts

To determine the influence of MRP1 on drug sensitivity of tumors *in vivo*, $MRP1^{+/+}$ and $MRP1^{-/-}$ murine neuroblastoma tumor cells (5×10^6/mL) were resuspended (1:1) with ice-cold Matrigel basement membrane matrix and the cell suspension (0.2 mL) was injected s.c. into both flanks of mice, which had been anesthetized with isoflurane. The complete details for the generation of $MRP1^{+/+}$ and $MRP1^{-/-}$ tumors are available in Supplementary Materials and Methods. Mice were monitored daily for 1 week following injection and tumors were measured every second day using a vernier caliper. Tumor mass was calculated as described previously (17, 18).

Evaluation of *In vivo* Toxicity for Reversan Alone and in Combination with Vincristine

The toxicity of single doses of Reversan (25, 50, and 100 mg/kg formulated in DMSO) was evaluated via i.p. injection using BALB/c mice. Mice were monitored >50 days for changes in body weight, mortality, and morbidity.

To determine whether Reversan altered the toxicity profile of vincristine, 7-week-old female BALB/c mice ($n \geq 5$ per dose per treatment group) were treated for 5 consecutive days with vincristine (0.05-0.4 mg/kg) alone or in combination with 10 mg/kg Reversan. As a positive control, a parallel set of mice was treated with vincristine in combination with 10 mg/kg cyclosporin A, a general multidrug transporter inhibitor. Mice were observed for signs of toxicity for up to 14 days. Mice were weighed daily for 1 week after the start of treatment. Mice in poor condition (e.g., sunken eyes, ruffled fur, and inactivity) or with >20% weight loss were euthanized before 14 days.

In vivo Chemotherapeutic Treatment

Following the development of a palpable tumor, *hMYCN* transgenic mice ($n \geq 10$) were treated daily for 5 consecutive days with single i.p. injections of vincristine or etoposide in the presence or absence of 10 mg/kg Reversan (i.p.). Saline and DMSO served as vehicle controls. Mice receiving vincristine alone or in combination with Reversan were supplemented with paraffin oil (100 μL) on days 0, 3, and 5 plus 0.1 mg/mL glutamic acid in the drinking water for 7 days to prevent severe constipation associated with vincristine in mice. Mice were monitored by experienced technical staff for evidence of tumor progression by twice weekly abdominal palpations and for visible changes in overall condition.

For $MRP^{+/+}$ or $MRP^{-/-}$ allografts ($n \geq 7$ mice per treatment group), cytotoxic drug treatment commenced as described above when tumors reached ~100 to 125 mm^3 in size. Mice were sacrificed when at least one of its tumors reached 2.5 times its starting mass, which was indicative of tumor progression. The time to reach 2.5 times the starting volume was calculated and the saline control was subtracted to give a growth delay

factor. For all efficacy studies, mice were treated with 0.2 mg/kg vincristine and 6 to 12 mg/kg etoposide depending on the mouse strain.

To determine whether Reversan exacerbated chemotherapy-induced neutropenia, blood was collected via the saphenous vein of BE(2)-C tumor-bearing mice treated with either 9 mg/kg etoposide alone or in combination with 10 mg/kg Reversan on day 15 following commencement of treatment, which is the nadir of WBC count following etoposide treatment (19). The complete blood count was evaluated using a Ac.T diff hematology analyzer (Beckman Coulter). To ascertain the WBC count composition, whole blood slides were stained with H&E, coverslipped, and counted using light microscopy.

Statistical Analysis

Two-tailed unpaired Student's t tests were used to test significance between groups, unless otherwise stated. Fisher's exact test was used to compare the survival of mice treated with vincristine in the presence or absence of modulating agents. Survival analysis was done using Kaplan-Meier (20) and two-sided log-rank tests. P values < 0.05 were considered statistically significant.

Results

Genetic deficiency of *MRP1* enhances tumor sensitivity to chemotherapeutic drugs. To determine the overall contribution of MRP1 to drug resistance in neuroblastoma, we crossed mice lacking the *MRP1* gene (*MRP1*$^{-/-}$) with human *hMYCN* transgenic mice, which develop neuroblastoma characteristic of the human disease. This cross yielded murine neuroblastoma tumors that were either wild-type (*MRP1*$^{+/+}$) or homozygous null (*MRP1*$^{-/-}$) for *MRP1* and thus contained high levels of MRP1 or entirely lacked MRP1, respectively (Fig. 1*A*). Tumor cells of either *MRP1* genotype were isolated and established as allografts in nude mice. Tumor-bearing mice were treated daily for 5 days with vincristine or etoposide, which are routinely used for the treatment of neuroblastoma patients and are also known MRP1 substrates, and monitored for tumor growth. The results of these studies are presented in Fig. 1*B*. Loss of MRP1 significantly increased the latency of tumor progression in response to both drugs. In contrast, the lack of MRP1 had no effect on the efficacy of cisplatin or cyclophosphamide, which are not substrates for MRP1 (data not shown). These data show that MRP1 is a major determinant of the response of neuroblastoma tumors to chemotherapy.

Identification of compounds inhibiting MRP1. Having established a direct link between MRP1 and the sensitivity of neuroblastoma tumors to conventional chemotherapy, we focused on the identification of small-molecule inhibitors of this multidrug transporter. As a readout system, we used MRP1-overexpressing cells that contained a p53-responsive reporter for the detection of a p53 response resulting from the DNA damage induced by the accumulation of doxorubicin, a known MRP1 substrate, in cells (Supplementary Fig. S1). Through our screening efforts, we identified six chemical scaffolds that functioned as putative MRP1 inhibitors (Supplementary Table S1; Supplementary Fig. S2). The most prominent and selective scaffold identified from the entire screening process (e.g., screening A and B) was the pyrazolopyrimidine scaffold, which is represented by CBLC4H10, CBLC4E10, and CBLC4E11 (Fig. 2). In particular, the pyrazolopyrimidines appeared to have increased selectivity for MRP1 as shown by the increased accumulation of the fluorescent MRP1 substrate daunorubicin in MRP1-overexpressing MCF7/VP cells with little or no effect on MCF7 parental cells (Fig. 2). Similar results were obtained with another MRP1 substrate, calcein (Supplementary Fig. S3).

Ability of modulators to sensitize cells to MRP1 substrates. MRP1 confers resistance to doxorubicin, vincristine, and etoposide but not to cisplatin or paclitaxel (21). We therefore tested the three most active pyrazolopyrimidine molecules for their ability to sensitize MCF7/VP cells to this panel of drugs (Table 1). CBLC4H10, CBLC4E10, and CBLC4E11 clearly sensitized cells to vincristine (10- to 15-fold), etoposide (7- to 12-fold), and, to a lesser extent, doxorubicin (3- to 4-fold). In contrast, these molecules did not increase sensitivity to cisplatin and paclitaxel. These data suggest that the effects of these compounds can be attributed to specific inhibition of MRP1-mediated drug efflux in this cellular context.

The selectivity of the pyrazolopyrimidines for MRP1 in terms of modulating drug response was further examined by studying the effects of CBLC4H10 on cell lines overexpressing one of several other multidrug transporters, including Pgp, MRP2, MRP3, MRP4, or MRP5. CBLC4H10 did not sensitize MRP2-, MRP3-, MRP4-, or MRP5-overexpressing cell lines to known substrates of each of these transporters: vincristine (MRP2), etoposide (MRP3), or 6-mercaptopurine (MRP4 and MRP5). In contrast, CBLC4H10 significantly sensitized Pgp-overexpressing cells [BE(2)-C/CHCb(1)] to vincristine (P < 0.0001), indicating that this molecule was not purely MRP1 specific (data not shown).

Effects of modulators on cytotoxic drug response in tumor cell lines *in vitro*. We examined the effects of the pyrazolopyrimidines on the drug response of human neuroblastoma [BE(2)-C],

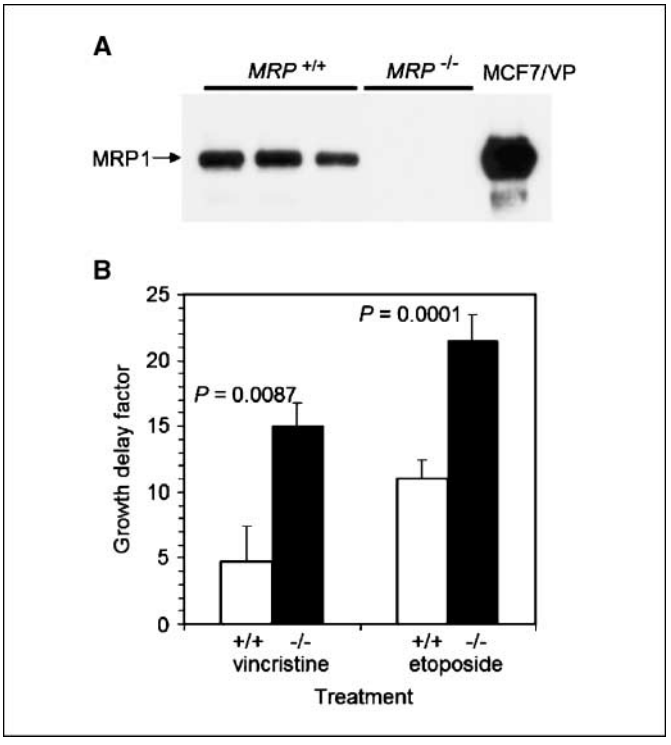

Figure 1. MRP1 expression in *MRP*$^{+/+}$ or *MRP*$^{-/-}$ murine neuroblastoma tumor cells from *MRP1*$^{+/+}$/*hMYCN* or *MRP1*$^{-/-}$/*hMYCN* transgenic mice. *A,* neuroblastoma tumors were harvested from *hMYCN* transgenic mice that were *MRP1*$^{+/+}$ or *MRP1*$^{-/-}$. Membrane proteins were purified from tumor cells and MRP1 levels were determined by Western blot. Representative samples from each *MRP1* genotype. MCF7/VP cells, which overexpress *MRP1*, were used as a positive control. *B,* effect of MRP1 status on the sensitivity of neuroblastoma cells to chemotherapeutic drugs. *MRP1*$^{+/+}$ or *MRP1*$^{-/-}$ murine neuroblastoma allografts were established in nude mice. Cytotoxic drug treatment [vincristine (0.2 mg/kg) and etoposide (12 mg/kg)] commenced when allografts were ~100 to 125 mg in size and continued for 5 consecutive days. The growth delay factor is the growth delay calculated from 2.5 times the tumor starting volume and normalized by subtracting the growth delay of the saline controls. The number of mice per treatment group is as follows: vincristine *MRP1*$^{+/+}$ (n = 8) and *MRP1*$^{-/-}$ (n = 7) and etoposide *MRP1*$^{+/+}$ (n = 20) and *MRP1*$^{-/-}$ (n = 17). *Bars,* SE.

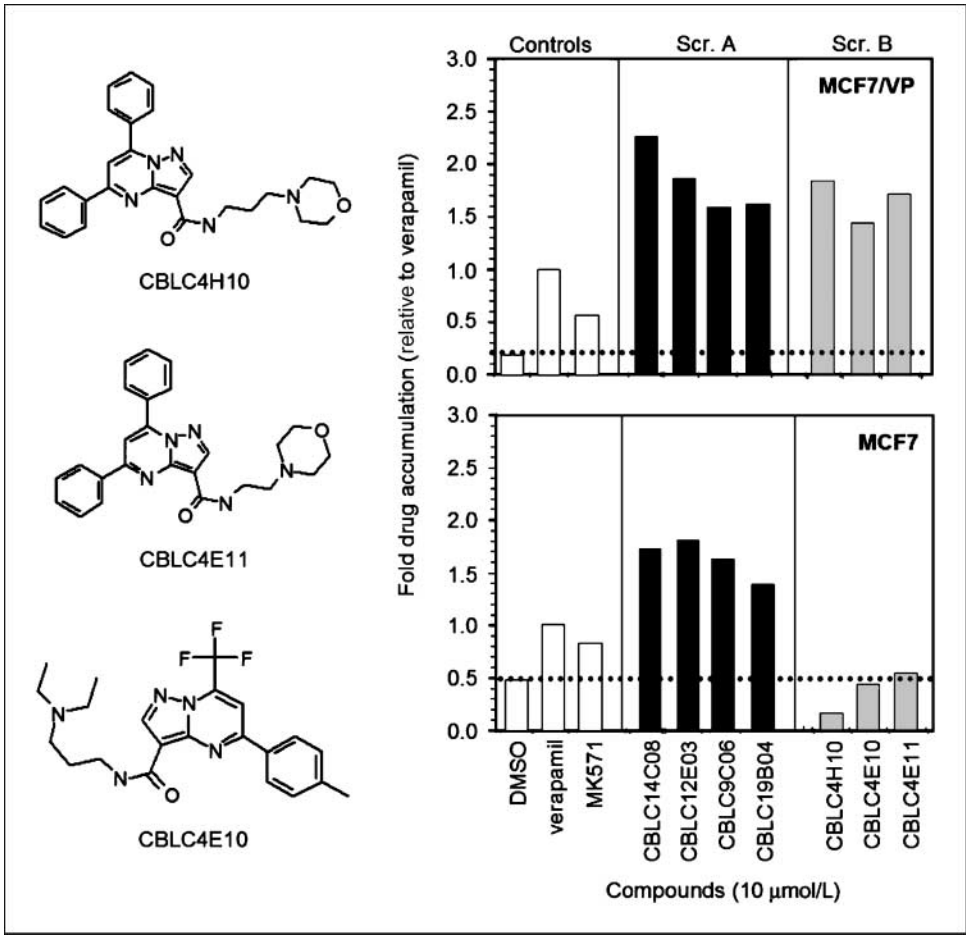

Figure 2. Fold accumulation of daunorubicin in parental MCF7 or MRP1-overexpressing MCF7/VP human breast cancer cells in the presence of hit compounds (structures of the pyrazolopyrimidine class of MRP1 inhibitors). Data are presented relative to verapamil controls. *Dotted line,* basal accumulation in the absence of any modulator; *black columns,* representative hits from screening A (*Scr. A*); *gray columns,* best compounds identified in screening B (*Scr. B*). A representative experiment is presented.

renal cell carcinoma (SK-RC45), and colon (HCT116) tumor cell lines, which represent tumor types that are clinically refractory to cytotoxic drug treatment as well as containing high levels of MRP1 protein (Fig. 3*A*). All three pyrazolopyrimidines caused increased sensitivity of these three drug-refractory tumor types to one or more cytotoxic drugs with the most dramatic effect observed in combination with vincristine (Fig. 3*B*). Importantly, neither of the compounds selected showed any effects, within the range of their solubility, on growth or viability of multiple cell lines tested (data not shown).

Based on our compiled *in vitro* data for the pyrazolopyrimidines, compound CBLC4H10 was chosen for further characterization and development because it was the most effective compound overall in increasing the sensitivity of drug-resistant MRP1-overexpressing tumor cells to conventional chemotherapeutic agents and thus has potential clinical importance as a modulator of MRP1. As such, this molecule has been named Reversan to exemplify its role in reversing drug resistance mediated by MRP1. Because Reversan represented a new class of MRP1 inhibitor, we compared the effect of Reversan on etoposide sensitivity in MCF7/VP cells to a panel of known drug transporter inhibitors, which included verapamil, cyclosporin A, difloxacin, probenecid, and PAK104P. Reversan increased the sensitivity of MCF7/VP cells to etoposide to a level similar to that of PAK104P (25-fold). More importantly, Reversan was six to eight times more potent than the rest of the panel of modulators, including the phase I clinical trial drug, probenecid (Supplementary Fig. S4). Thus, our compound is a potential lead for the development of a clinically relevant MRP1 reversal agent.

***In vivo* efficacy.** The toxicity of single i.p. injections of Reversan (25, 50, and 100 mg/kg) was tested in BALB/c mice. Due to the

Table 1. Fold sensitization of MCF7/VP cells to MRP1 substrates by pyrazolopyrimidines

Drug	Doxorubicin	Vincristine	Etoposide	Cisplatin	Paclitaxel
Verapamil	2.5	8.9	2.7	1.0	1.2
CBLC4H10	3.8	14.6	11.6	0.9	1.4
CBLC4E10	3.2	11.8	6.8	1.0	1.1
CBLC4E11	2.9	10.4	7.7	1.5	1.2

NOTE: Fold sensitization = (IC_{50} drug alone) / (IC_{50} drug + 5 μmol/L compound).

limited solubility of Reversan, 50 and 100 mg/kg doses were delivered in the form of a suspension. No adverse effects were observed for all three doses tested as measured by observations of general mouse appearance, mouse weight, and survival. Because the dissolution rate of Reversan from the suspension within the mouse is unknown, the toxicity of the 50 and 100 mg/kg doses can be underestimated to some extent. The 25 mg/kg dose, which appeared to be completely soluble, was clearly safe for single i.p. administration.

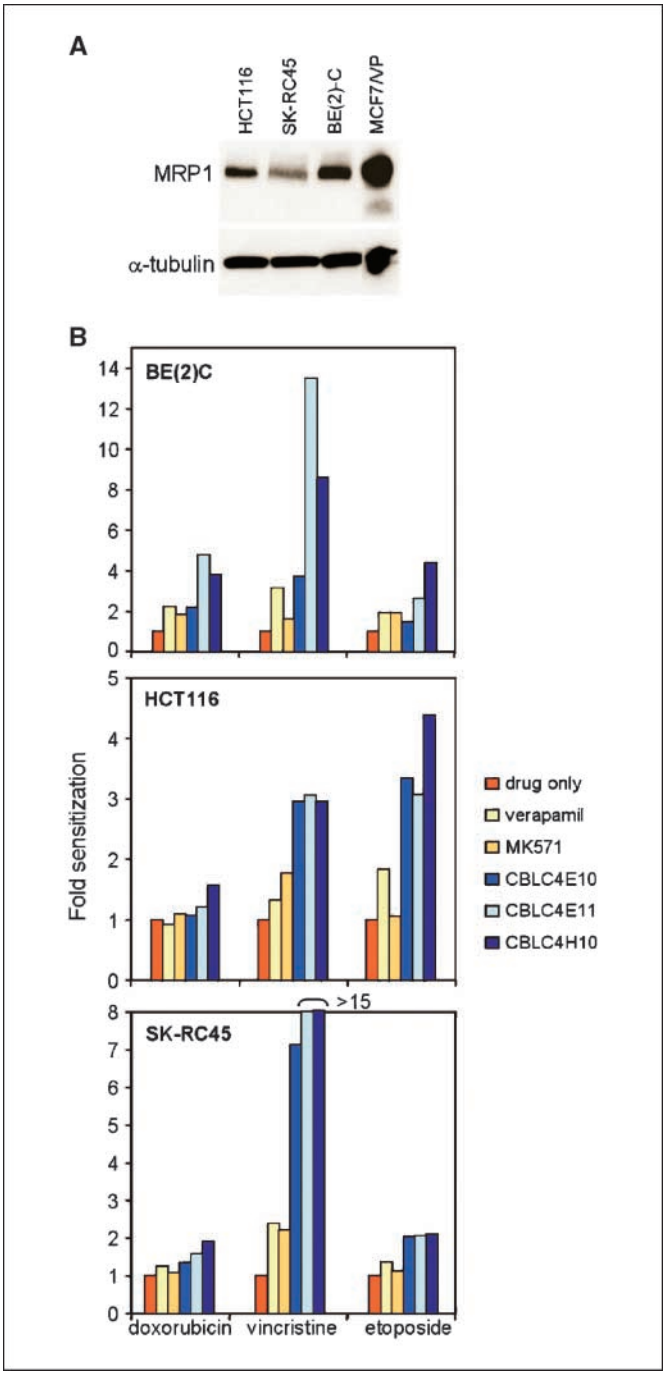

Figure 3. Effects of pyrazolopyrimidines on MRP1-expressing tumor cells. *A,* total cell lysates (20 μg) were isolated from each tumor cell line and separated by SDS-PAGE. Western blots were probed with a MRP1-specific antibody and anti-rat secondary antibody. MCF7/VP (2 μg) was included as a positive control. *B,* tumor cell lines were treated with a range of concentrations of doxorubicin, vincristine, or etoposide in the presence or absence of 10 μmol/L pyrazolopyrimidines. Verapamil and MK571 served as control transporter modulators. Data are presented as fold sensitization (IC_{50} drug alone / IC_{50} drug + modulator).

To determine whether Reversan could effectively increase the sensitivity of neuroblastoma tumors to conventional chemotherapeutic agents *in vivo*, we used a transgenic mouse model of this disease. On the development of a palpable tumor, *hMYCN* transgenic mice were treated daily for 5 consecutive days with vincristine or etoposide alone or in combination with 10 mg/kg Reversan. Mice were then monitored until signs of tumor progression were evident. The combination of Reversan with vincristine (Fig. 4*A*) or etoposide (Fig. 4*B*) significantly increased the survival time of mice compared with those treated with drug alone. Whereas treatment with vincristine alone increased survival of tumor-bearing *hMYCN* mice by ~10 days, the addition of Reversan to the vincristine treatment regimen increased survival an additional 20 days (survival: 4.9 ± 0.49 days saline control, 16.2 ± 0.89 days vincristine alone, and 36.5 ± 4.4 days vincristine + Reversan). For treatment with etoposide alone, the duration of survival of tumor-bearing *hMYCN* mice doubled compared with vehicle control and tripled when coadministered with Reversan (survival: 4.9 ± 0.49 days saline control, 11 ± 0.67 days etoposide alone, and 16 ± 0.56 days etoposide + Reversan). The combination of Reversan and cyclophosphamide, which is not a MRP1 substrate, had no effect on the duration of time between treatment and progression compared with cyclophosphamide alone (22.8 ± 2.2 days cyclophosphamide alone and 27.9 ± 2.0 days cyclophosphamide + Reversan; P = 0.115). Reversan also significantly increased the efficacy of vincristine and etoposide against BE(2)-C human neuroblastoma xenografts (Supplementary Fig. S5). It should also be noted that oral administration of Reversan worked equally as well as i.p. administration for increasing the efficacy of etoposide administered to tumor-bearing *hMYCN* mice.

Effect of Reversan on vincristine toxicity. Past attempts at modulating multidrug resistance have failed mainly due to nonspecific side effects that became apparent when modulators were combined with conventional drugs (22). To determine whether Reversan increased the toxicity profile of vincristine, BALB/c mice were treated with vincristine [0.05-0.4 mg/kg, which includes doses in the clinically relevant range for this drug (23)] in the presence or absence of 10 mg/kg Reversan or 10 mg/kg cyclosporin A, a first-generation multidrug transporter inhibitor that underwent clinical trials in the 1990s (24, 25). All mice were followed for up to 14 days for signs of toxicity, such as weight loss, general poor health, or lethality. We found that Reversan did not significantly alter the toxicity profile of vincristine (Fig. 4*C*). In stark contrast, vincristine administered in combination with 10 mg/kg cyclosporin A resulted in rapid weight loss and a dramatic shift in the toxicity profile of vincristine, such that only 0.05 mg/kg vincristine could be safely given without any adverse side effects. Neither Reversan nor cyclosporin A was toxic when administered as individual drugs. Importantly, there was no toxicity associated with Reversan at clinically relevant doses of vincristine. Similar results for Reversan were obtained for both males and females in a second mouse strain, ICR, which shows that lack of toxicity was not a gender-specific or strain-specific artifact (data not shown).

Effects of Reversan on the complete blood count of etoposide-treated mice. To further evaluate the effect of Reversan on the toxicity of conventional chemotherapeutic agents, we tested BE(2)-C xenograft-bearing nude mice for signs of hematopoietic

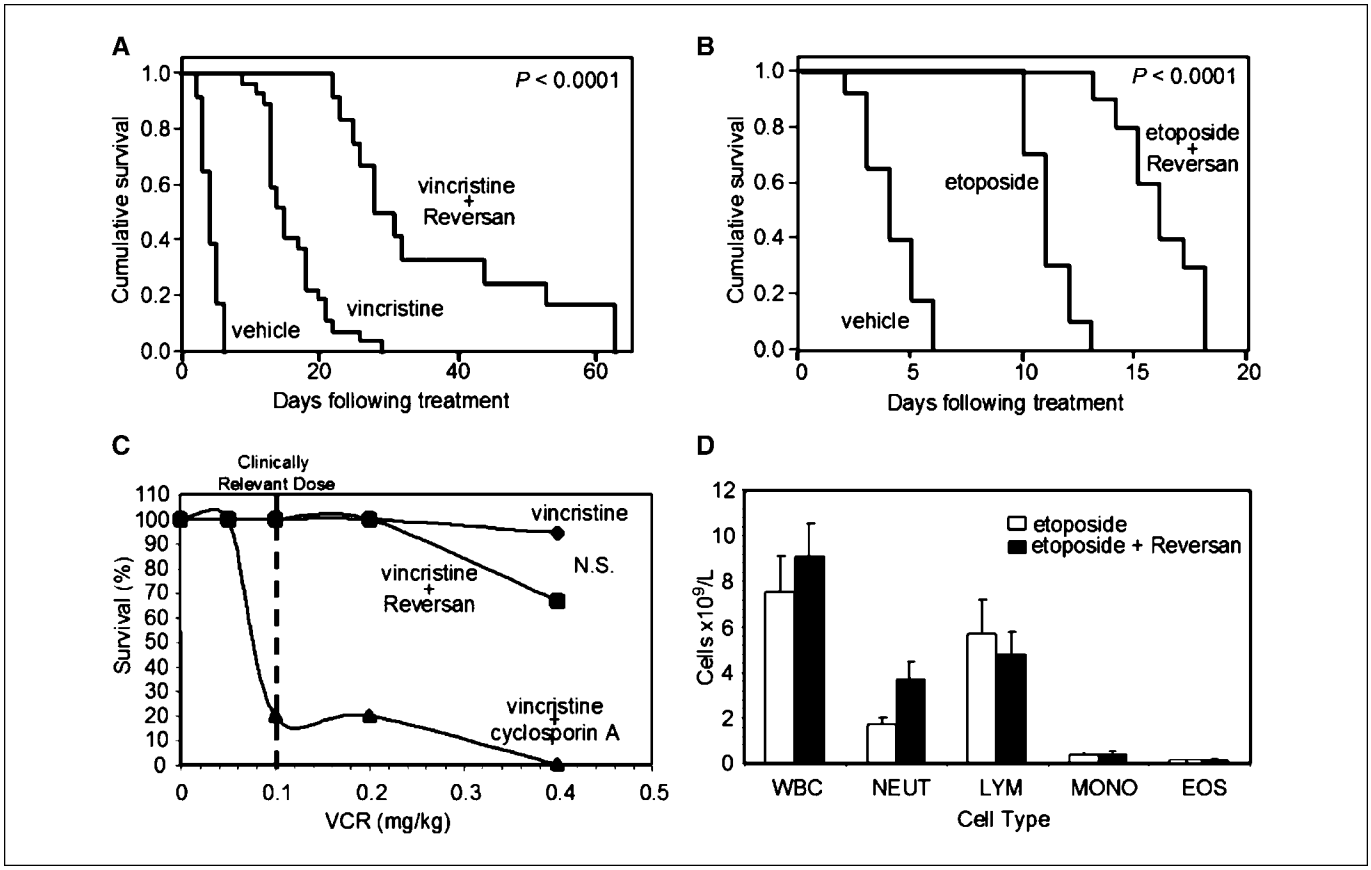

Figure 4. Efficacy and safety of Reversan. On development of a tumor, *hMYCN* transgenic mice were treated with conventional chemotherapeutic agents [0.2 mg/kg vincristine (*A*) or 6 mg/kg etoposide (*B*)] in the presence or absence of 10 mg/kg Reversan daily for 5 consecutive days. Mice were followed until signs of tumor progression were evident. *C,* 7-week-old female BALB/c mice [$n = 5$ per dose per treatment group, except for 0.4 mg/kg vincristine (*VCR*) ± 10 mg/kg Reversan, where $n = 18$ per treatment group], were treated i.p. with 0.05 to 0.4 mg/kg vincristine for 5 consecutive days in the presence or absence of 10 mg/kg Reversan or 10 mg/kg cyclosporin A and observed for signs of toxicity for up to 14 d. Mouse survival per vincristine dose is presented. *D,* blood was collected from BE(2)-C tumor-bearing nude mice following treatment with 9 mg/kg etoposide ± 10 mg/kg Reversan and analyzed for blood cell parameters, including total WBC, neutrophils (*NEUT*), lymphocytes (*LYM*), monocytes (*MONO*), and eosinophils (*EOS*).

toxicity following treatment with etoposide alone or in combination with Reversan on day 15, the nadir of WBC count following etoposide treatment (19). There was no effect of Reversan on the number of lymphocytes, monocytes, eosinophils, or basophils (Fig. 4*D*). However, there was a significant increase (~2-fold; $P = 0.027$) in the number of neutrophils in mice treated with the drug combination compared with mice treated with etoposide alone. Thus, Reversan appeared to have a protective effect on the neutrophil population and does not enhance etoposide-induced neutropenia. There was no significant effect of the etoposide/Reversan combination on the platelet population (data not shown).

Discussion

One of the major problems in the fight against cancer is the intrinsic or acquired resistance of tumors to current cancer treatments. Although many mechanisms of resistance exist, the majority of clinical and experimental data indicate that multidrug transporters, which keep intracellular drug concentrations low (e.g., Pgp and MRP1), play a leading role in treatment failure (8). Thus, inhibition of the function of these drug efflux pumps in tumor cells presents one of the most promising approaches to cure cancer using existing chemotherapy. This approach is not a new strategy; however, the clinical failures of early Pgp inhibitors significantly diminished the enthusiasm of drug developers. Nevertheless, drug resistance associated with multidrug transporters remains a serious problem and the development of inhibitors against these proteins must be revisited using the knowledge and experience gained from past failures to increase the likelihood of success. For instance, first-generation Pgp inhibitors had lower affinity to Pgp than to other target proteins (e.g., verapamil and Ca^{2+} channel) and thus could not be administered at sufficiently high doses to inhibit Pgp without causing toxicity through high-affinity targets (8). Second-generation Pgp modulators, although more specific, had the potential to change the pharmacokinetics of conventional chemotherapy agents by altering their metabolism and clearance from the body presumably through interactions with cytochrome *P*450 (e.g., valspodar; refs. 26–28), which often shares substrates with Pgp. Therefore, such inhibitors competed with the conventional drugs for metabolism as well as for efflux (29, 30).

Currently, third-generation Pgp inhibitors (e.g., tariquidar and zosuquidar) have reached various stages of clinical investigation (8). These molecules are potent, highly specific Pgp inhibitors that are not themselves substrates of this transporter (31–33), which suggests that pharmacokinetic interactions may not be an issue. Indeed, early clinical trials showed that tariquidar could reverse drug efflux in patients (34, 35) and be coadministered with paclitaxel, vinorelbine, or doxorubicin without the need for dose reduction of the

chemotherapeutic agents (8). It should be noted that several, more recent clinical trials with tariquidar have been prematurely ended (phase II or III in breast and lung cancer) due to low efficacy or increased toxicity in the tariquidar containing treatment arm (36). Therefore, optimization of dose, scheduling, and drug combinations are still required and additional phase II clinical trials are ongoing. In clinical trials with zosuquidar, only modest pharmacokinetic drug interactions have been observed to date and at the same time increased antitumor activity of conventional agents in previous drug-refractory tumors (37–40). Therefore, multidrug transporter modulators have renewed clinical potential.

Although the present study was focused predominantly on neuroblastoma, MRP1 has been associated with poor treatment response not only in neuroblastoma but also in both non-small cell and small cell lung cancer, breast cancer, and prostate cancer (41). Thus, identification of clinically relevant inhibitors of MRP1 would have broad applications in the treatment of drug-refractory tumors. In this report, we have established conclusively that MRP1 plays a role in resistance to conventional chemotherapy *in vivo*. Consequently, we set out to identify MRP1 inhibitors that were safe by themselves and did not significantly affect the pharmacokinetic properties of conventional agents, two critical characteristics based on the history of Pgp inhibitor development. We have found such an inhibitor in Reversan. Because the majority of "failed" Pgp inhibitors (first- and second-generation) were substrates and competed with the conventional agents for transport by Pgp and possibly CYP3A4, we reasoned that, if we could identify inhibitors of MRP1 that were not themselves substrates of the transporter, we might increase the likelihood of identifying molecules that did not interfere with the pharmacokinetics of the conventional agents. Therefore, we hypothesized that screening of a library of compounds selected around the structural features of previously identified Pgp modulators, which were determined not to be Pgp substrates, would lead to the identification of MRP1 inhibitors with novel mechanisms of action, that is, nonsubstrate/noncompetitive inhibitors of MRP1 function. We have independently confirmed that Reversan is not a substrate for MRP1 by showing that similar levels of [^{3}H]Reversan accumulate in MCF7 and MCF7/VP cells despite the increased level of MRP1 in the latter cell line due to gene amplification. Currently, the exact mechanism of action of Reversan and related pyrazolopyrimidines is unknown. Because the original Pgp modulators around which the screening library was generated are not ATPase inhibitors, it is likely that the pyrazolopyrimidines are also not inhibitors of ATPase, but this remains to be tested. It is possible that Reversan does not interact directly with MRP1 or Pgp but rather alters the physicochemical properties of the membrane surrounding the transporter, which could then alter the structure of the protein(s) within the membrane and affect its ability to transport. Indeed, it appears that the hydrophobic nature of Reversan and the other similarly active pyrazolopyrimidines may be important for their potency because more hydrophilic pyrazolopyrimidine analogues (logP <4 versus 4.92 for Reversan) were less effective in reversing drug resistance (Supplementary Table S1).

Although some critics of this field have suggested that inhibitors of multidrug transporters should be specific for individual transporters or toxicity would be increased due to inhibition of off-target transporters, the results of the current study suggest that this is not the case for Reversan. Despite the fact that Reversan inhibits the function of both MRP1 and Pgp equally well, it does not significantly alter the toxicity profile of conventional chemotherapeutic agents *in vivo* in contrast to cyclosporin A, which exemplifies the earlier generation of toxic multidrug transporter modulators. Perhaps the problem of off-target toxicities is not related to interactions with other transporters but more related to interplay between Pgp and CYP3A4 in terms of shared substrates (29, 30). The increase in the effectiveness of conventional chemotherapeutic agents observed with Reversan in the absence of an increased toxicity of these conventional agents indicates that Reversan has an excellent therapeutic index compared with multidrug inhibitors of the past. Thus, it appears that Reversan represents a new class of "safe" multidrug transporter inhibitor that may be clinically useful in the treatment of neuroblastoma and other cancers associated with aberrant MRP1/Pgp expression.

Disclosure of Potential Conflicts of Interest

M.D. Norris: ownership interest, Cleveland BioLabs. A.V. Gudkov: consultant and Board of Directors, commercial research grant, and ownership interest, Cleveland BioLabs. M. Haber: ownership interest, Cleveland BioLabs. The other authors disclosed no potential conflicts of interest.

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